

## THE EFFECTIVENESS OF DIFFERENT ISOTHIOCYANATES ON ATTRACTING LARVAE OF *PIERIS RAPAE*

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THE EFFECTIVENESS OF BLACK MUSTARD OIL (allyl-isothiocyanate) on attracting larvae of *Pieris rapae* at various concentrations has already been described in this JOURNAL (Hovanitz and Chang, 1963). This mustard oil is the commonly used oil for condiments and is prepared by pressing from the seeds of black mustard (*Brassica nigra*). This oil is known also from various species of Cruciferous plants in addition to black mustard. Other mustard oils are known from a wide variety of plants, not all of them members of the family Cruciferae. This paper describes some experiments on the selection by *Pieris rapae* larvae on allyl-isothiocyanate produced by two different methods, and on three other isothiocyanates.

### NATURALLY OCCURRING ISOTHIOCYANATES

Isothiocyanates (mustard oils) are naturally derived from glucosides which occur in a wide variety of the higher plants, belonging to a relatively small number of plant families (Kjaer, 1960). These glucosides are characterized by the ability to undergo enzymatic hydrolysis to isothiocyanates, hydrogen sulfate and D-glucose. The latter has invariably been encountered as the sugar moiety of the more than thirty individual compounds recorded thus far (Kjaer, 1960). According to this author, only nine glucosides of these thirty isothiocyanates have as yet been crystallized. The occurrence of more than one glucoside in a given plant species is most common; as many as eight individual glucosides have been distinguished in a single seed specimen. The compounds appear to be distributed over the entire plant; the glucosides are diffusely present in parenchymal tissues, especially in

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the bark (Guignard, 1890, 1893). The embryo constitutes the site of accumulation in seeds. Little has been done to relate the variation in glucoside content as a function of the stage of growth, or of environmental factors such as climate, soil, etc. Stahmann, et al (1943) found that 2-phenylethylisothiocyanate, the aglucone of gluconosturtiin, to be the predominant mustard oil enzymatically liberated from the roots of *Brassica nigra*, whereas the seeds of this plant represent the classical source of the glucoside sinigrin which yields allyl isothiocyanate upon enzymatic fission. Delaveau (1958) has noticed considerable variation in the total and relative amounts of the individual glucosides in the plant *Alliaria officinalis* during its growth cycle. Considerable variation has been detected in quantitative and qualitative differences in glucoside content of various parts of a plant as well as in different lots of the same plant tissue.

A list of the known natural isothiocyanates and their parent glucosides in various plant genera and species which are known to be hosts of species of *Pieris* is given in table 1. In this table the plants are listed by families and genera. After each genus or species of plants is listed (1) the *Pieris* known to feed on it, (2) the mustard oil glucoside which has been isolated, or established as having been found, in that plant, (3) the aglycones (the mustard oils corresponding to the glucoside) and (4) the group of compounds to which it belongs.

Observation of this list does not seem to indicate any common denominator between the mustard oil, or glucoside, and the species of *Pieris* which is attracted to it, with a single exception. That is, sinigrin is present in most plants attracted by one or more species of *Pieris*. This, in itself, may mean little for sinigrin appears to be the most common mustard oil glucoside, and the earliest known. There are *Pieris* attracted by plants in which sinigrin is not known, for example, (1) *Lepidium*, which is a common food plant of *Pieris protodice* and *P. occidentalis*, (2) *Cleome* (and probably *Isomeris*), which is a common food plant of *P. protodice*, and *P. beckeri*, (3) *Tropaeolum*, a food plant of *Pieris rapae*, and (4) various *Resedaceae*, food plants of *Pieris daphnidice*. In each of these plants indicated, there are some other mustard oil glucosides present, namely, glucotropaeolin and glucoraphanin for *Lepidium*, glucotropaeolin for *Tropaeolum*, gluconasturiin, glucobarbarian, and glucotropaeolin for the *Resedaceae*, and glucocapparin for *Cleome* (or *Isomeris*, *Capparidaceae*).

It seems likely that more than one mustard oil attracts *Pieris*, even of the same species. The possibility exists that there may be little specificity for the kind of mustard oil, so long as some one isothiocyanate is present in the proper concentration. Except for *Tropaeolum*, all listed food plants of *Pieris rapae* contain sinigrin in some part of the plant. Only glucotropaeolin is known from *Tropaeolum* itself, yet *Pieris rapae* survives on that plant after only a few generations of adaptation.

## TESTS OF LARVAE OF PIERIS RAPAE TOWARD VARIOUS ISOTHIOCYANATES

The tests to be described here were made by subjecting larvae of *Pieris rapae* from our laboratory strain, grown for several generations on black mustard (*Brassica nigra*), to our testing device. This device has been described previously (Hovanitz and Chang, 1963). It consists essentially of a greenhouse flat, with filter paper moistened with dilutions of mustard oils, and spaced equally around the periphery of the flat. The larvae are set in the center and make their way to the side of the flat, being attracted to one or another of the mustard oils, at various concentrations.

For this experiment, tests were made of five different mustard oils in concentrations from  $10^{-5}$  to  $10^{-9}$ . These five were:

- (1) benzyl-isothiocyanate: this is the aglycone of glucotropaeolin, found in Cruciferae: *Lepidium* sp., *Resedaceae*, and Tropaeolaceae: *Tropaeolum* sp. Synthetic origin.
- (2) phenyl-isothiocyanate: there is no natural aglycone of this mustard oil listed by Kjaer. Synthetic origin.
- (3) phenethyl-isothiocyanate: this is the aglycone of gluconasturtiin, found in *Barbarea*, *Brassica nigra*, *Brassica oleracea* and the *Resedaceae*. Synthetic origin.
- (4) allyl-isothiocyanate: this is the aglycone of sinigrin found in many plants. Commercial synthetic.
- (5) same as above, only commercial natural product from *Brassica nigra* seeds.

Sources of the above mentioned mustard oils are described below.

### General Procedure for the Preparation of Synthetic Mustard Oils

The following general procedure for the preparation of mustard oils from various amines was provided by Professor Henry Klostergaard of the San Fernando Valley State College. This method was used for the preparation of the phenethyl-, benzyl-, and methyl-isothiocyanates used in this paper. All procedures to be carried out at 0°C.

In a 2000 ml. Erlenmeyer flask, mix 0.07878 moles of the amine and enough absolute ethyl alcohol to make a 30% solution (by volume). To the above solution add 4.8 ml. CS<sub>2</sub> solution (0.03939 moles in a 1:1 solution with absolute alcohol). Wait 10 to 15 minutes, stirring often as there will be some heat evolved. Now add 100 ml. of the iodine solution (0.03939 moles or 10 gms. of iodine dissolved in 100 ml. absolute alcohol). The solution will start to clear up. Disregard any crystallization at this point. Shake until the brown color is gone and some yellow color appears. This is not stable so immediately go on to the next step. At this point add 25 ml. of the sodium solution (0.03939 moles or 0.9 gms. of sodium in 25 ml. absolute alcohol). The mixture will split and become homogeneous.

Now add another 100 ml. of the iodine solution as above. Some elementary sulfur will settle out (this is not soluble in ether). Now add 250 ml. acidified water (1% HCL).

Next pour the solution into a separatory funnel and extract the mustard oil with ether. The mustard oil will appear in the ether phase or the top

TABLE 1. The occurrence of natural isothiocyanates and their parent glucosides in plant genera and species known to be hosts of *Pieris* (chemical data from Kjaer).

<u>PLANT</u>	<u>INSECT</u>	<u>GLUCOSIDE</u>	<u>MUSTARD OIL</u>	<u>ISOTHIOCYANATE GROUP</u>
CRUCIFERAE				
<u>Alyssum</u>	<u>Pieris rapae</u>	sinigrin glucoberteroin glucoalysin gluconapin glucobrassicinapin	allyl isothiocyanate 5-Methylthiopentyl isothiocyanate 5-Methylsulphinylpentyl isothiocyanate 3-Butenyl isothiocyanate 4-Pentyl isothiocyanate	unsaturated alkyl isothiocyanates ( <i>A</i> )-Methylthioalkyl " unsaturated alkyl " ( <i>A</i> )-Methylthioalkyl
<u>Arabis</u>	<u>Pieris sisymbrii</u> <u>Euchloe</u> sp.	glucoarabin	9-Methylsulphinylhomyl isothiocyanate	"
<u>Barbarea</u>	<u>Pieris napi</u>	glucohirsutin glucocochlearin glucoputranjivin gluconapin sinigrin	8-Methylsulphinyl octyl isothiocyanate ( <i>A</i> )-2-Butyl isothiocyanate isoPropyl isothiocyanate see above see above	" saturated alkyl isethiocyanates " " "
<u>Brassica nigra</u>	<u>P. rapae</u> <u>P. protodice</u> <u>P. occidentalis</u>	glucocapparin gluconasturtiin glucobarbarin sinigrin	Methyl isothiocyanate 2-Phenylethyl isothiocyanate 2-Hydroxy-2-phenylethyl isothiocyanate see above	" aromatic isothiocyanates hydroxy-substituted "
<u>Brassica oleracea</u> var. <u>capitata</u>	<u>P. rapae</u>	gluconasturtiin	see above	"
<u>Brassica oleracea</u> vars.	<u>P. rapae</u>	glucorapiferum	2-Hydroxy-3-butenyl isothiocyanate	"

<u>Brassica oleracea</u> vars. cult.	<u>P. nani</u>	glucosiferum	2-Hydroxy-3-butenyl isothiocyanate	( <i>L</i> )-Methylthioalkyl isothiocyanates
		glucomasturtiin	see above	
		glucobrassicinapin	see above	
		glucosajin	see above	
		sinigrin	see above	
		glucobarin	3-Methylsulphinylpropyl isothiocyanate	
		glucocheirolin	3-Methylsulphonylpropyl isothiocyanate	
		glucoibervirin	3-Methylthiopropyl isothiocyanate	
	<u>Pteris nari</u>	not specific		
	<u>P. protodice</u>	glucotropasolin	benzyl isothiocyanate	aromatic isothiocyanates
	<u>P. occidentalis</u>	glucoraphanin	4-Methylsulfinyl-butyl isothiocyanate	( <i>L</i> )-Methylthioalkyl isothio- cyanates
		glucolepidin	ethyl isothiocyanate	saturated alkyl isothiocyanate
		sinalbin	p-Hydroxybenzyl isothiocyanate	aromatic isothiocyanates
	<u>Raphanus</u>	glucoraphanin	see above	
		sinigrin	see above	
	<u>Sisymbrium</u>	<u>P. protodice</u>	isopropyl isothiocyanate	saturated alkyl isothiocyanate
		<u>P. occidentalis</u>	2-Hydroxy-isopropyl iso- thiocyanate	Hydroxy-substituted "
		<u>P. beckeri</u>	(+)-2-Butyl-isothiocyanate	
			see above	
CAPPARIDACEAE	<u>Cleome</u> also probably <u>Isomeris</u>	<u>P. protodice</u>	methyl isothiocyanate	saturated alkyl isothiocyanate
		<u>P. beckeri</u>		
RESERACEAE		<u>P. depidice</u>	see above	
		glucobarbarin	2-Hydroxy-2-phenylethyl isothiocyanate	Hydroxy-substituted isothiocyanate
		glucotropasolin	see above	
TROPAEOLACEAE		<u>P. rapae</u>	glucotropasolin	benzyl isothiocyanate
Tropaeolum				aromatic isothiocyanates

layer. Discard the lower layer. Add some 10% NaOH to the mustard oil and ether solution to take off the iodine and then discard the lower layer. Next wash the solution with an equal volume of distilled water. Discard the water layer. Now there is present an ether solution of the mustard oil. (Ice this solution immediately.) Vacuum distil this solution in a flash evaporator with the solution in the flask cooled by an ice-salt solution and successive flasks cooled by ether and dry ice. Keep the mustard oil and ether solution under vacuum for at least  $1\frac{1}{2}$  hours. The mustard oil will be left in the original flask. Remove it immediately and store at 0°C.

#### Caution

All the solutions involved in this preparation should be kept refrigerated at 0°C. until used. All work should be done under an adequate hood since the chemicals involved are highly toxic and odoriferous.

Tests of the larvae of *Pieris rapae* as indicated were made separately, utilizing the five dilutions of the mustard oils indicated. The actual dilutions may actually have been less than indicated because it appears that at the temperature used (about 20°C.), not all the mustard oil may have gone into solution. Each larva was tested twenty times, giving total test times ranging from 180 times for benzyl-isothiocyanate to 680 times for phenyl-isothiocyanate.

The allyl-isothiocyanates differed in their response, according to their origin. One was derived from black mustard seeds by standard commercial techniques of compression, and fermentation. The other was synthetically prepared. In each case, the larvae selected the  $10^{-6}$  dilution of mustard oil (22.1% for synthetic to 21.2% for natural) but the dispersal of the selections was different. For example, the dilution of  $10^{-5}$  was selected by the larvae for the synthetic mustard oil 20.7% of the time, as compared with only 11.6% for the natural product. Dilutions of less than  $10^{-7}$  are also different. For example, while the synthetic gave selections of 19.3% at  $10^{-8}$ , the natural product gave 14%. At  $10^{-9}$ , these were 15.2% for the synthetic as compared with 11.6% for the natural. An apparently significantly greater number of larvae left the flat without any selection at all with the natural (24.2%) as compared with the synthetic product (2.5%). This discrepancy was probably due to the human variation in carrying out the tests. The percentage is greater when the larvae are not allowed as long a selection period. This discrepancy would not affect the actual selections themselves.

Another effect is quite noticeable with these data, as well as with the data on the other mustard oils, namely, that the selections give a bimodal curve (see fig. 1). Such a bimodal curve was not clear with our previous testing though a trace of it might be detected (fig. 3, Hovanitz and Chang, 1963). The previous testing was made with the natural product which also shows a very poor bimodal curve (fig. 1). The synthetic product shows a more strongly indicated bimodality at  $10^{-6}$  and at  $10^{-8}$  (fig. 1). The reasons for the bimodal curve which is especially apparent for the synthetic allyl mustard oil are unknown.

Selections for benzyl-isothiocyanate are almost certainly negative,

TABLE 2. The selection by larvae of *Pieris rapae* for various concentrations of mustard oils.

	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>	10 <sup>-8</sup>	10 <sup>-9</sup>	distilled water	no selection	no. of larvae	total trials
Benzyl-isothiocyanate	16 8.9%	15 8.3%	11 6.1%	15 8.3%	25 13.8%	17 9.4%	81 45%	9	180
Phenyl-isothiocyanate	101 14.4%	127 18.7%	80 11.8%	118 17.4%	81 11.9%	83 12.2%	91 13.4%	34	680
Phenethyl-isothiocyanate	60 15.0%	83 20.8%	48 13.5%	76 19%	65 18.8%	27 6.8%	25 6.3%	20	400
Allyl-isothiocyanate (synthetic)	116 20.7%	124 22.1%	56 10.0%	108 19.3%	85 15.2%	57 10.2%	14 2.5%	28	560
Allyl-isothiocyanate natural	58 11.6%	106 21.2%	54 10.8%	70 14%	58 11.6%	33 6.6%	121 24.2%	25	500

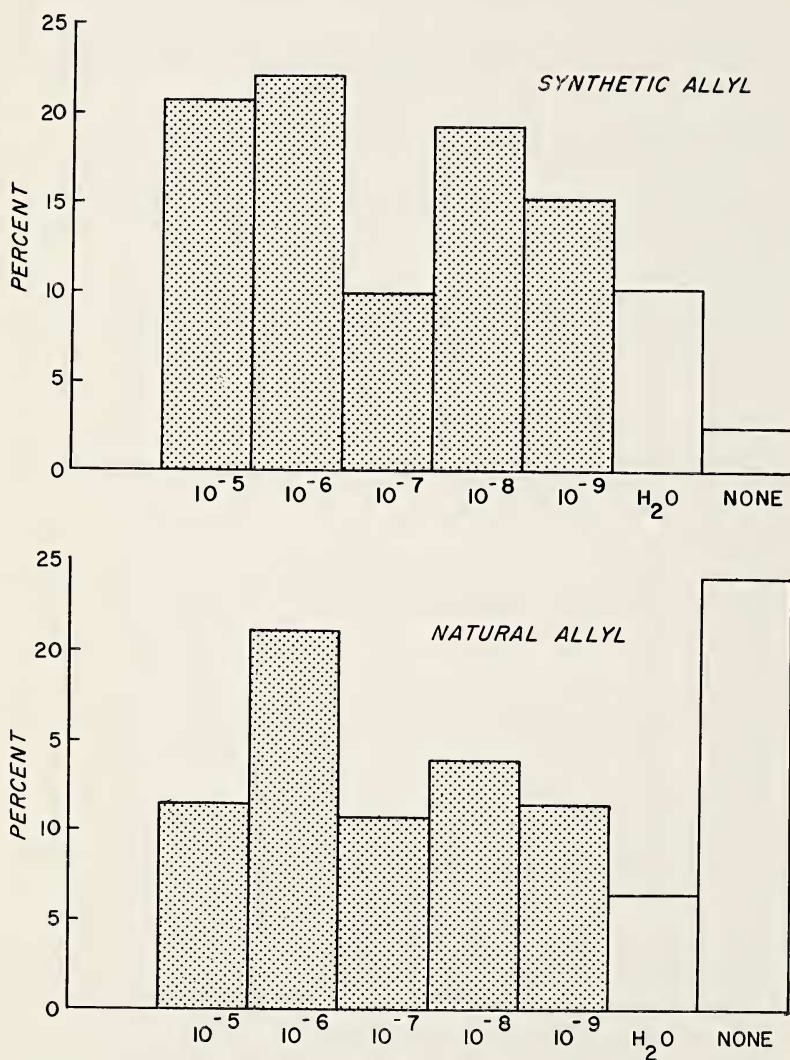


Fig. 1. Histograms illustrating the comparison between natural and synthetic mustard oils respecting their selection by larvae of *Pieris rapae*. Note the strong bimodality of the histogram showing the synthetic product as compared with the natural.



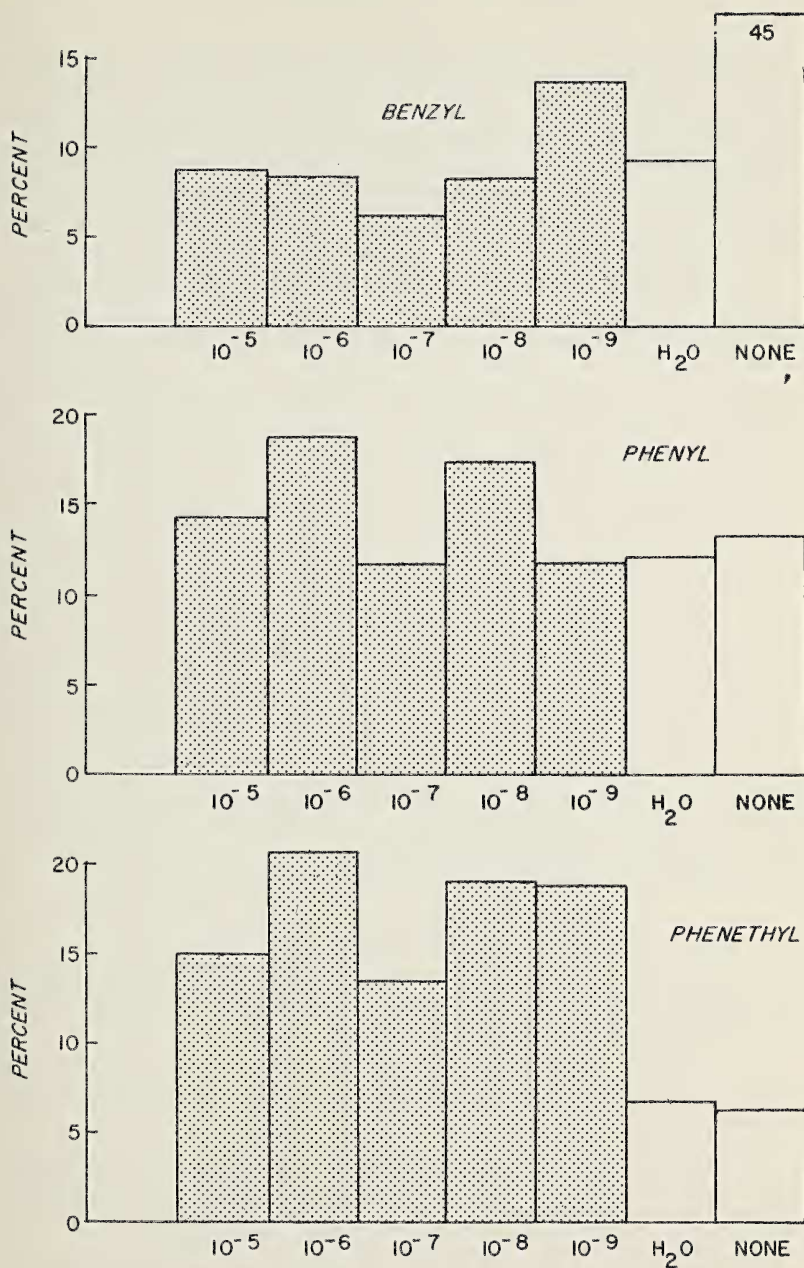


Fig. 2. Histograms illustrating the selections made by larvae of *Pieris rapae* for various dilutions of the mustard oils benzyl-isothiocyanate, phenyl-isothiocyanate and phenethyl-isothiocyanate. Note the bimodality of two of the histograms and the nearly complete lack of selection indicated by the other.

not varying significantly from water alone (table 2 and fig. 2). This mustard oil also was the one in which dissolving in water appeared almost impossible and in which case the tests might very well have been conducted on water alone. Perhaps at a higher temperature, dissolving the mustard oil might have been accomplished but under the circumstances it does not seem as if this mustard oil could be of any influence on attracting *Pieris rapae* larvae to *Tropaeolum* species where it is found naturally.

Phenyl-isothiocyanate and phenethyl-isothiocyanate were about equally effective in attracting the larvae though there is a possibility that the phenyl mustard oil may be slightly less attractive than the phenethyl, since at each concentration the percent attraction was slightly less. However, the decrease possibly is not significant. The attraction of both of these mustard oils was not much different from that of allyl isothiocyanate (table 2 and fig. 2). This leads to the interesting possibility of the specificity between isothiocyanates in the generic sense and the Pieridae that are attracted to plants that contain the compounds. It is possible that the attraction is to the isothiocyanate part of the molecule and not to side chains. "When applied to the tongue, all mustard oils cause a sharp and burning sensation. Their odors, though mostly pungent, display characteristic individual differences which often are helpful in the detection and classification of mustard oils. Certain isothiocyanates, that undergo rapid intramolecular cyclization, give rise to a transient biting taste, followed by a sensation of bitterness. Like most synthetic mustard oils, those of natural origin show vesicant and frequently also lachrymatory properties," from Kjaer (1960). These same substances, however, when present in small concentration appear to give rise to the desirable condiment properties of the mustard oils and appear in turn account for the specificity of these oils to the larvae and adults of *Pieris*.

### CONCLUSIONS AND SUMMARY

1. A list is given of the mustard oils which are known to occur in food plants of the Pieridae. These are derived from naturally-occurring glucosides which are also listed.
2. It seems evident from this list that more than one mustard oil attracts *Pieris*, since the evidence does not indicate that only one could be responsible.
3. Tests were made on the selection of larvae of *Pieris rapae* toward several mustard oils at various concentrations. These oils were either commercially obtained, or were synthetically compounded by us. The procedure used for synthetic compounding is described.
4. Synthetic and natural allyl mustard oils obtained commercially gave somewhat divergent testing results. The synthetic gave a bimodal curve with modes at  $10^{-6}$  and  $10^{-8}$  which were only slightly

or not at all apparent with the natural.

5. Other mustard oils tested were benzyl-isothiocyanate, phenyl-isothiocyanate and phenethyl-isothiocyanate.

6. Benzyl-isothiocyanate is the aglycone of glucotropaeolin, the glucoside of *Tropaeolum* sp. and some other plants. Its attraction power at the concentrations used is not different from that of distilled water. In view of the difficulties experienced in dissolving this substance, it is possible that none actually dissolved and that, therefore, the tests were actually on water alone.

7. Phenyl-isothiocyanate is not known to be natural occurring. Nevertheless, it had a selective effect on the larvae only slightly less effective than allyl- and phenethyl-isothiocyanates.

8. Phenethyl-isothiocyanate had an attractive power almost equivalent to that of the synthetic allyl-isothiocyanate. It is a naturally-occurring mustard oil, being the aglycone of gluconasturtiin which is found in *Nasturtium* and *Barbarea*.

9. It appears that more mustard oils than just allyl-isothiocyanate are attractive to *Pieris*. The experience with benzyl-isothiocyanate however indicates that not all are effective, at least not under the experimental conditions utilized.

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